NITROXIDES XLVIII: A STUDY OF THE INTERACTION BETWEEN BOVINE SERUM ALBUMIN AND A MODIFIED STEROID BY ELECTRON SPIN RESONANCE

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1. Introduction

Although most experimental biochemical methods have been used in the study of protein–steroid interaction [1], spin-labelling methods [2], which should provide more direct information, have not to our knowledge been used for this type of study, probably because of the lack of stable spin-labelled steroids. The recent preparation of steroidal nitroxide (see structural formula (1)) [3] now allows such a study.

Two kinds of information can in principle be obtained: (1) structural and chemical data on the surroundings of the steroid, and (2) thermodynamic and kinetic data on the equilibrium between bound and free steroid.

This should lead to a better understanding of the binding site properties and ultimately its structure.

As a first example, we have choosen [4] a non-specific [5] protein: bovine serum albumin (BSA). We wish to report quantitative results on the binding equilibrium of this with steroidal nitroxide (1).

2. Materials and methods

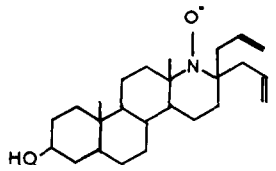
Steroidal nitroxide (1) (MW = 386) has been prepared by Ramasseul and Rassat [3]. BSA was purchased from Sigma (96–99%) and used as such.

In the competitive binding experiment, 4 identical samples were prepared: to 40 μl of BSA (120 g/l, in 0.15 M phosphate buffer pH 7.4) was added 1/25 μCi (ca. 3 ng) of ^{3}H-progesterone in 2% ethanol saline.

To this solution (a), steroidal nitroxide in 2% ethanol saline was added in following quantity: (b) 200 ng; (c) 500 ng; (d) 5000 ng. The mixtures were then equilibrated 10 min at 37° and 45 min at 4°.

Free and bound steroids were separated by adsorption of the free form onto charcoal–dextran and centrifugation. Counting of the supernatant liquid (bound ^{3}H-progesterone) was carried out with a Packard liquid scintillator counter.

Electron spin resonance (ESR) spectra were recorded on a Varian E 3 spectrometer equipped with a variable temperature accessory. For the experiments, BSA solutions were obtained in water. The steroidal nitroxide concentration was kept constant: 0.25 g/l (5 μl of an ethanolic nitroxide solution, 10 g/l, added to 0.2 ml of protein solution). The incubation period was 30 min at 30° after the solution was homogeneous.



(1)

Structural formula 1.

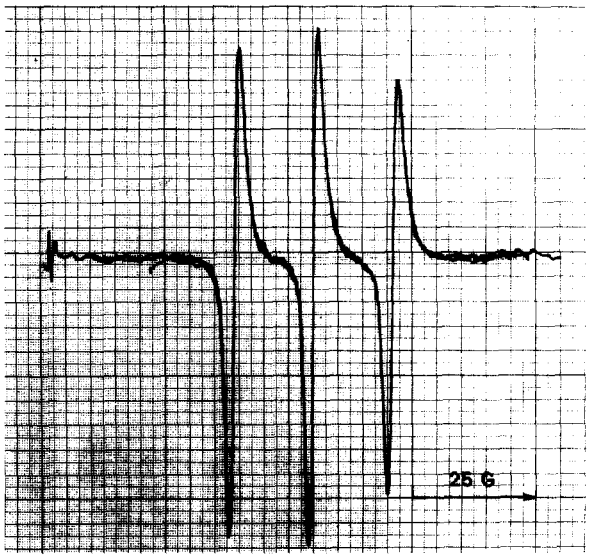


Fig. 1. ESR spectra of steroidal nitroxide (1).

3. Results

3.1. *Competitive binding experiments*

At high concentration, steroidal nitroxide displaces tritium labelled progesterone from BSA. Activity of the supernatant solution drops from 2244 dpm for (a) to 1732 for (b), 1680 for (c) and 1420 for (d). This is characteristic of non-specific binding [6].

3.2. *Electron spin resonance studies*

Fig. 1 shows the ESR spectrum of steroidal nitroxide (1) in ethanol/water (1:1, v/v) at a concentration of 10^{-2} g/l. The intensities of the two external lines measured on this spectrum have been used as a standard for quantitative measurements.

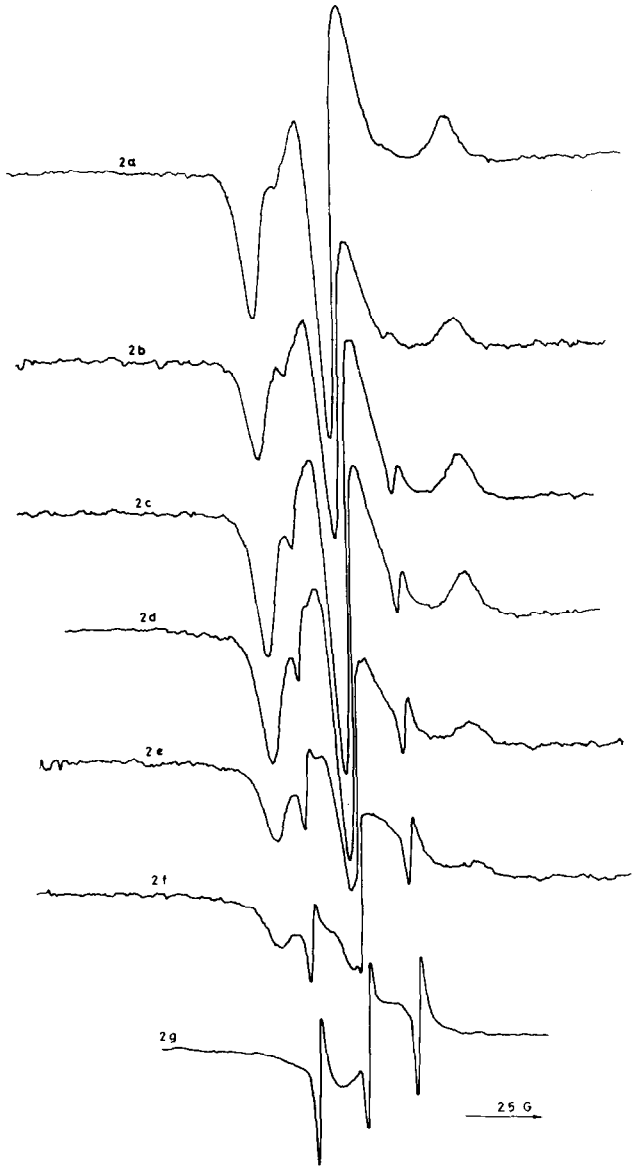
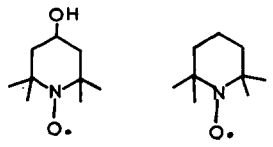


Fig. 2. Concentration dependence of BSA – steroidal nitroxide spectra. a = 240 g/l; b = 120 g/l; c = 90 g/l; d = 60 g/l; e = 30 g/l; f = 10 g/l; g = steroidal nitroxide (1) in water.

Concentration dependence at 30°. Fig. 2 shows the ESR spectra obtained from a 0.2 ml solution of BSA in water at varying concentrations: a. 240 g/l, b. 120 g/l, c. 90 g/l, d. 60 g/l, e. 30 g/l, f. 10 g/l, in the presence of 50 μg of steroidal nitroxide.



(2) (3)

Structural formulas 2, 3.

Spectrum 2a is assigned to "strongly immobilised" steroidal nitroxide [7]. In the other spectra (2b to 2e), two new bands can be observed superimposed onto the "strongly immobilised" spectrum. These two new bands are assigned to free steroidal nitroxide in water. The intensity of these two bands is proportional to the free steroidal nitroxide concentration.

Spectrum 2f is slightly different: the sample tube was no longer clear, and a third superimposed spectrum was assigned to the precipitated steroid phase. Such a precipitation can be observed when 5 μl of the steroidal nitroxide solution is added to 0.2 ml of water (fig. 2g). At all larger concentrations of BSA, dissolution is complete [8].

On fig. 2, the anisotropic nitrogen coupling constant A_{zz} [7] can be measured: $2A_{zz}$ = 64.5 G. Since we found for nitroxide (1): $2A_{zz}$ = 69.5 G in ethanol glass at -160° and 65 G in o-terphenyl glass at -140°, and 64.5 G in o-terphenyl at room temperature*, steroid (1) must have its D ring in a *non-polar* environment, i.e. in a hydrophobic binding site.

Under the same conditions, the smaller nitroxide molecules [9] tanol and tanane (see structural formulas (2) and (3), respectively) give rise to two superimposed spectra [4]: one assigned to free nitroxide in water, and the other one to an almost free nitroxide in a *non-polar* site. Quantitative data on this binding equilibrium between BSA and various small non-steroidal nitroxide ligands will be reported in future publications.

Association constant. We have calculated the West-

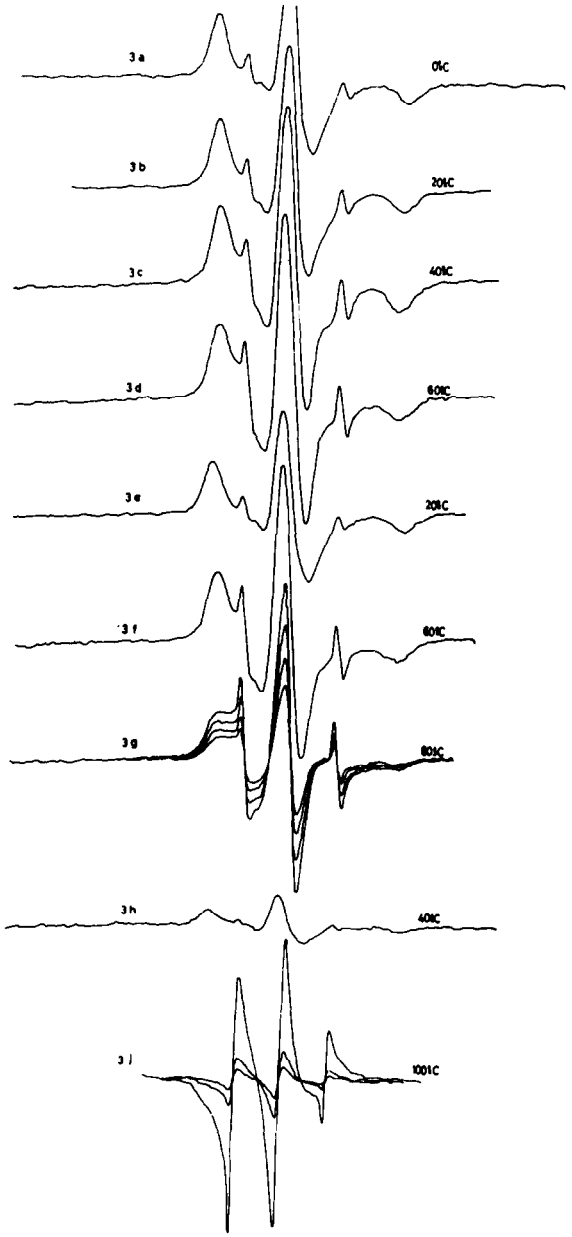


Fig. 3. Temperature dependence of BSA – steroidal nitroxide (1) spectra.

* This variation is consistent with the known nitrogen splitting dependence on solvent polarity [9]. For temperature dependence of the hyperfine splitting in a similar steroidal nitroxide, see [10].

phal combining affinity constant C [11]:

$$C = \frac{[Sbd]}{[S]\ [P]}$$

in which the concentration of the protein–steroid complex [Sbd], unbound steroid [S], free protein [P] in the solution are expressed in g/l.

Because of the order of magnitude of the concentrations used [P] = [P_0], initial concentration of BSA; [Sbd] = [S_0], initial concentration of steroid. [S] is taken as proportional to the peak to peak intensity of the unbound nitroxide, calibrated against fig. 1. Average of 10 independent determinations gives: $C = 2.5 \pm 0.5$ l/g at 30°, or, since the molecular weight of steroid is 386, nk = $0.9 \pm 0.2 \times 10^4$ M^{-1} at 30°.

Temperature dependence. Using a solution of steroid (1) (50 μg) in 0.2 ml of a 60 g/l BSA solution, we have recorded the ESR spectra at various temperatures (fig. 3) in the following order: a. 0°, b. 20°, c. 40°, d. 60°, e. 20°, f. 60°, g. 80°, and h. 40°. Except for spectrum 3g, a stationary signal was reached in ca. 10 min. Between 0° and 60°, the percentage of unbound steroid increases reversibly with temperature (spectra 3a–f). At 80° the signal decays. Simultaneously, the solution in the sample tube becomes white. Fig. 3g shows the signal decay by continuous recording of the total spectrum (2 min sweep). The central part of the spectrum is reduced by half in ca. 8 min. Spectrum 3h at 40° shows that this decay is irreversible. Similar behaviour is observed at 200°: fig. 3j shows continuous recording of another identical sample (2 min sweep). The correlation time of the bound steroid is now estimated to be ca. 10 nsec [7] and the central part of the spectrum is reduced by half in ca. 2 min.

It must be observed that nitroxide (1), although stable at room temperature, decays at 80°. The signal intensity of this nitroxide in water is reduced by half in ca. 25 min at 80° and in ca. 5 min at 100°. Its faster decay in the presence of BSA at 80° and 100° may be ascribed to an attack of the nitroxide by reducing agents (probably R–SH, since nitroxides are reduced by thiols [12]).

Another information is obtained from this temperature-dependence study: as the temperature increases, the total width of the spectrum decreases; the bound steroid correlation time becoming smaller.

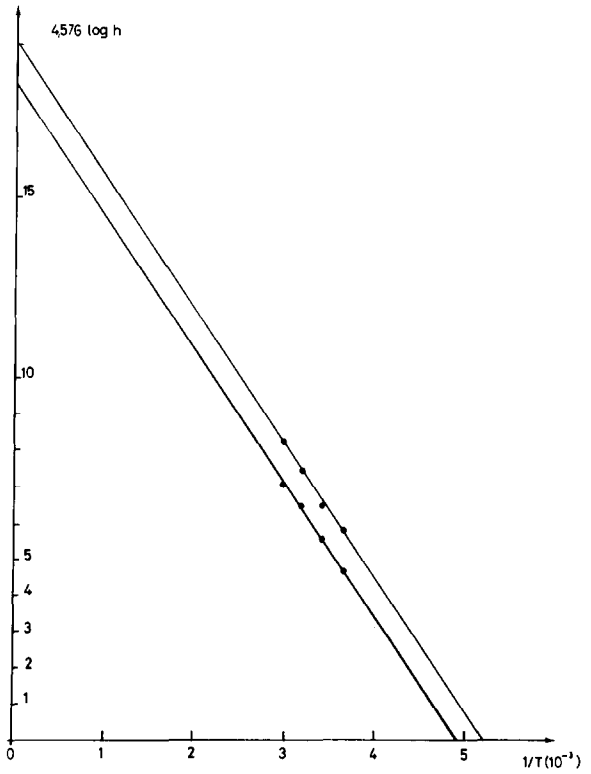


Fig. 4. Arrhenius plot for BSA/steroidal nitroxide (1) equilibrium.

Since it is reasonable to assume that the correlation time of BSA in water is larger than 100 nsec, even at 80° [13], the decrease of correlation time of steroid (1) with temperature must be assigned to an increasing mobility in the binding site. The observed phenomena at 80° and 100° are in agreement with the Kautzman proposed mechanism [14, 15] for denaturation of protein: as temperature increases, the conformational mobility in the binding site increases and an irreversible opening of the disulfide bridge occurs.

Enthalpy and entropy of association of BSA with steroid (1) in water. In the temperature range where reversibility is observed (0–60°), an Arrhenius plot (fig. 4) gives ΔH and ΔS. In fact ΔH can be directly measured by plotting $\log h_1$ and $\log h_2$ versus $1/T$; h_1 and h_2 being the height of the two unbound steroid peaks. It is found that: $\Delta H = -3.9 \pm 0.1$ kcal × $mole^{-1}$ and $\Delta S = +0.90 \pm 0.2$ cal $K^{-1} \times mole^{-1}$.

These values are similar to those measured for other steroids [1].

4. Conclusion

These experiments show that steroidal nitroxide (1) behaves as most of the biologically active hormonal steroids relative to a non-specific protein: reversible binding occurs between 0 and 60° with a small entropy of association and an enthalpy of association of ca. 4 kcal/mole. The binding site is non-polar, and mobility of this steroid nitroxide in its binding site increases with temperature.

These experiments demonstrate the usefulness of spin labelling technique in the study of protein–steroid interaction. This method is more flexible than others for the study of some binding characteristics (e.g. temperature dependence or variation with chemical modification [16]). Binding parameters (e.g. C) can be readily obtained without the need for a separation between the free and the bound forms of the steroid. Thermodynamic data and structural information are thus directly available and compare favorably with those obtained by other methods.

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